Alterations in Hepatic Cholesterol Levels in Response to Drugs That Induce Cytochrome P450 3A23

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Abstract: In this study, hepatic cholesterol levels were examined after treatment of rats with a series of structurally distinct drugs that induce the P450, CYP3A23. It was found that these agents decreased levels of unesterified and esterified cholesterol in microsomes, but generally increased levels of these lipids in crude membrane fractions from the rest of the liver. Troleandomycin induces CYP3A23 but is also an efficacious mechanisms-based inhibitor of this enzyme, so that treatment with this agent decreases overall CYP3A activity. Similar to other CYP3A23 inducers, treatment with troleandomycin suppressed cholesterol levels, suggesting that the effects on cholesterol are unlikely to be dependent upon increased CYP3A23 activity. Rather, it seems more likely that cholesterol processing within the liver is modified as a result of activation of a receptor, such as the pregnane X receptor, which regulates several enzymes involved in cholesterol metabolism as well as CYP3A23 expression.

Key words: Cholesterol, cytochrome P450, nicardipine, pregnane X receptor

INTRODUCTION

In mammals, cholesterol and cholesterol esters are generally obtained in the diet or synthesized in the endoplasmic reticulum of liver cells. Most of the cholesterol formed in the liver is either converted to a bile acid or esterified and released systemically[1]. (Although the term cholesterol is commonly used clinically to refer to the combination of unesterified and esterified cholesterol, in this manuscript cholesterol specifically refers to unesterified cholesterol, while total cholesterol refers to the combination of esterified and unesterified cholesterol). Hepatic rates of synthesis and release of total cholesterol are key factors in hypercholesterolemia, a condition which is associated with increased risk of vascular diseases, heart failure and stroke. Similarly, lipid trafficking in the liver is an important factor in defining the circulating levels of total cholesterol, but the mechanisms regulating intracellular cholesterol transport are generally poorly understood[3,5]. Cellular cholesterol is present in two pools, commonly referred to as detergent soluble and insoluble fractions. The insoluble cholesterol fraction is associated with lipid rafts, which contain concentrated levels of glycosylphosphatidylinositol-anchored proteins and other proteins important in cell-signaling and disruption of this fraction may be important in the development of Niemann-Pick type C disease[6-8].

Cytochrome P450 (P450) is a superfAMILY of proteins involved in the oxidation of a wide variety of lipophilic compounds. Some P450s specifically catalyze the formation of cholesterol precursors and the conversion of cholesterol to steroids and bile acids.[2,9]. In contrast to these highly specific catalysts, enzymes in three P450 families, CYP1, CYP2 and CYP3, have broad substrate and catalytic capabilities, but are generally not involved in anabolic metabolism[10]. Members of these three "drug-metabolizing" P450 families tend to be concentrated in the hepatic endoplasmic reticulum, can be highly inducible and contribute to the breakdown and elimination of a diverse group of pharmaceutical, dietary and endogenous compounds.

There is evidence that levels of the drug-metabolizing P450s and the levels of cholesterol are interdependent. DNA microarray analyses demonstrated that mice lacking the CYP1A2 gene exhibit a general disruption in the cholesterol biosynthetic pathway[11]. In turn, intermediates in the cholesterol and bile acid synthetic pathways can induce the expression of CYP2B and CYP3A gene subfamilies.[12,13] CYP3A induction is likely to be mediated primarily by the pregnane X receptor (PXR), which has a central role in preventing excess accumulation of cellular steroids and bile acids[8,14]. CYP3A's protective role results from its ability to hydroxylate many steroids and bile acids[6,11,14].

PXR can be activated by a variety of drugs as well as steroids and bile acids, a point that suggests that drugs that induce CYP3A may have unexpected effects on cholesterol regulation. Potentially, these effects could
result from either increased P450 activity or by non-P450 proteins whose expression is co-regulated by the same receptor. In this study, we examined if drugs that induce CYP3A expression also altered hepatic cholesterol levels in rats. Since such effects may be mediated by changes in cholesterol synthesis in the endoplasmic reticulum as well as alterations in subcellular trafficking, we examined cholesterol levels in microsomal fractions as well as crude membrane preparations that lacked the microsomal lipids. It was found that treatment with CYP3A inducers reduced cholesterol and total cholesterol levels in microsomes but increased levels of these lipids in other crude membrane fractions. Even so, these effects were unlikely to be dependent upon increases in CYP3A activity, suggesting that these effects were the result of induction of proteins that are co-regulated by the PXR.

**MATERIALS AND METHODS**

**Materials:** Chemicals and enzymes were from Sigma Chemical Company (St. Louis, MO), unless noted otherwise. An anti-peptide antibody specific for CYP3A23 has been described previously[19]. Anti-CYP4A and anti-CYP2B were from BD Biosciences (Bedford, MA) and Oxford Biomedical (Oxford, MI), respectively. Secondary antibodies conjugated to horseradish peroxidase were from Jackson Laboratories (West Grove, PA).

**Animals:** Six-week-old male Sprague-dawley rats were used in all studies. Animals were treated and sacrificed as described by Zhang et al.[16,17]. In general, rats were given either a control diet, or the same diet containing 100 mg kg⁻¹ of nicardipine, nimodipine, diltiazem, clotrimazole, or pregnenolone 16α-carbonitrile, 50 mg kg⁻¹ ketocamazole, or 20 mg kg⁻¹ ciprofibrate each day for 7 days (unless noted otherwise). Alternatively, for the three-day time course study rats were treated by gavage with either 100 mg kg⁻¹ nicardipine or, for the control animals, with vehicle. In the case of dimethylsulfoxide (DMSO), rats were given a 1 mL intraperitoneal 12 h prior to sacrifice[16]. Hepatic membrane fractions that represented microsomes, nonidet P-40 (NP40)-soluble and NP40-insoluble fractions were prepared[14,19] and were stored at −80°C prior to use. In brief, liver homogenates were centrifuged at 10,000 g and the pellet and supernatant collected. This supernatant was then centrifuged at 100,000 g and the washed pellet was used as the microsomal fraction. To obtain the NP40-soluble and NP40-insoluble fractions, the 10,000 g pellet was washed with detergent-free buffer and then incubated with 0.5% NP40, a nonionic detergent. The 10,000 g supernatant from this fraction was isolated and used as the NP40-soluble fraction. The pellet, which was suspended by sonication in 1% lauryl sulfate, was the NP40-insoluble fraction.

**Cholesterol Assays:** Cholesterol levels were measured by two methods: gas chromatography with mass spectrometric detection (GC MS) or a fluorescence assay. For GC MS, aliquots (250 µg protein) of the subcellular fractions were diluted to 250 µL with 0.1 M NaPO₄, pH 7.4. The lipids were extracted using diethyl ether, as describe previously[19]. Following evaporation of the diethyl ether phase under a gentle stream of nitrogen gas, the sample was suspended in 100 µL of ethyl acetate and derivatized with 75 µL of N-methyl-N-trimethylsilyl trifluoroacetamide for 2 h at room temperature[19]. Derivatized samples were separated and detected using an HP6890 gas chromatograph equipped with an HP5973 mass selective detector and a RTX-SMS column from Restek (Bellefonte, PA) that was 30 m x 0.25 mm internal diameter x 0.25 df. The oven had an initial temperature of 150°C for 1 min, ramped at 25°C min⁻¹ to 200°C, then ramped at 25°C min⁻¹ to 300°C and held for 7 min. Total run time was 12 min. The injection inlet was in splitless mode at a constant temperature of 210°C. Head pressure was a constant 25 psi and the column gas was helium. Sample volumes of 1.5 µL were injected into a Restek 4 mm internal diameter cyclo-double gooseneck injection liner.

Cholesterol standards of 0, 10, 20, 40, 70 and 100 µM were prepared in duplicate in 250 µL of 0.1 M NaPO₄, and were extracted, processed and analyzed in parallel with the biological samples. Integration of a single-ion mass peak (m/z of 329), which eluted at 10.5 min, was used to quantitate the cholesterol. Linear standard curves were generated with this method with correlation coefficients (r²) of 0.95 or higher. In the biological samples, comparisons of the spectrum from the corresponding peak with a mass spectra database (from the National Bureau of Standards) confirmed that this peak was derivatized cholesterol.

In order to measure total cholesterol, samples were first extracted with ether, as described above, then the cholesterol esters were hydrolyzed to cholesterol[20]. In brief, the ether-extracted lipids were allowed to air dry for 1 h. The lipids were then resuspended in 60 µL of 8.9 M KOH and 500 µL of 100% ethanol and incubated at 65°C for 1 h, with occasionally mixing. One hundred and twenty five microliter water and 375 µL HPLC-grade hexanes (Fisher Scientific, Pittsburgh, PA) were added to each sample prior to vortexing for 3 min and centrifugation at 10,000 g for 2 min. The upper phase, which contained the neutral lipid fraction, was collected and dried under vacuum. The samples were then derivatized and analyzed the same as described above. Duplicate samples of 0, 10,
20, 40, 70 and 100 μM cholesterol palmitate in 250 μL of 0.1 M NaPO₄ were prepared, extracted, processed and analyzed in parallel with the biological samples. Although cholesterol palmitate was used as the standard for total cholesterol, initial tests with cholesterol and several cholesterol esters demonstrated that any of these compounds would produce equivalent results.

Cholesterol was also assayed using fluorescence detection[23]. In brief, 0.5 mg microsomal protein was brought to 1 mL with reaction buffer (0.1 M NaPO₄, pH 7.4, 10 mM sodium cholate, 3 mM p-hydroxyphenylacetic acid, 6 U mL⁻¹ horseradish peroxidase and 0.6 U mL⁻¹ cholesterol oxidase). Samples were then incubated for 60 min in the dark and analyzed (Ex. 320, Em. 400) using a Fluorolog spectrofluorometer (JY Horiba, Edison, NJ). Addition of various cholesterol esters to the assay did not alter the background readings. Therefore, this assay appears to be specific for unesterified cholesterol.

**Immunoblot analyses:** Immunoblots were performed as described by Zangar et al.[23] Anti-CYP3A23, anti-CYP2B and anti-CYP4A were diluted 1:30,000, 1:7,500 and 1:30,000, respectively. Horseradish-peroxidase conjugated secondary antibody was used at 1:5000 dilution. Protein bands were imaged and quantitated by chemiluminescence using a Lumi-Imager F1 (Mannheim Boeringer, Indianapolis, IN).

**Statistical analysis:** Statistical comparisons were undertaken using SigmaStat 2.0 software (SPSS Science, Chicago, IL). A t test was used to compare levels of a single treatment group with the control group. Other statistical analyses were undertaken using a one-way ANOVA and, when appropriate, followed by a Tukey’s analysis. A probability value of <0.05 was used for all analyses.

**RESULTS**

Nicardipine is a calcium channel antagonist that activates the PXR, transcriptionally induces several P450s and it is an exceptionally efficacious inducer of CYP3A protein[11,23]. In contrast, DMSO transiently induces CYP3A, but apparently acts only at the level of protein stability[12,24]. Initial studies were undertaken to determine if these two P450 inducers affected cholesterol levels in rat liver. Nicardipine treatment decreased cholesterol levels in the microsomes (Fig 1), which are primarily composed of lipid membranes from the endoplasmic reticulum. In contrast, DMSO was without effect on microsomal cholesterol levels. In order to determine how rapidly treatment with nicardipine may alter microsomal cholesterol levels, rats were examined at 1, 2 and 3 days after initiation of treatment. It was found that cholesterol levels were decreased by ~50% following one day of treatment and effects appeared to be maximal after two days of treatment (Fig. 2). These results suggest that the effects of nicardipine treatment on cholesterol levels are rapid.

Since nicardipine is both a calcium channel antagonist and an efficacious P450 inducer, studies were undertaken to differentiate between these two possible mechanisms of action. Although nicardipine doses of

![Fig. 1: Effects of DMSO and nicardipine treatment on microsomal cholesterol levels. Each column and crossbar represents the Mean±SE, respectively of cholesterol levels in individual hepatic microsomal samples from four rats. *Values are significantly different from the control group (p<0.05)](image1)

![Fig. 2: Time-dependent effects of nicardipine treatment on microsomal cholesterol levels. Each time point and crossbar represents the Mean±SE, respectively of cholesterol levels in individual hepatic microsomal samples from four rats. *Values are significantly different from the control group (p<0.05)](image2)
5 to 15 mg kg⁻¹ have been reported to be sufficient to alter renal vascular resistance and sodium excretion rates in rats, higher doses are typically required for P450 induction. Dose-response studies indicated that nifedipine doses of 50 mg/kg/day or greater were necessary to alter microsomal cholesterol levels (Fig. 3). Next, two other calcium channel antagonists, nifedipine and diltiazem were examined. At the doses used, only nifedipine is an effective P450 inducer. Of these two agents, only nifedipine treatment effectively decreased cholesterol levels in microsomes (Fig. 4).

We subsequently examined the effects of a variety of structurally and functionally distinct agents at doses known to induce P450s. We found that treatment with pregnenolone 16α-carbonitrile, clotrimazole, troleandomycin and nifedipine all suppressed microsomal cholesterol levels from ~50% (Fig. 5a).

Induction of CYP3A23, CYP2B and CYP4A are indicative of activation of PXR, constitutive androstane receptor and peroxisome proliferator activated receptor, all of which could potentially have a role in the regulation of genes involved in cholesterol processing. Therefore, we use immunoblot analysis to determine the levels of these P450s in the same set of microsomal samples which exhibited drug-induced suppression of cholesterol levels. CYP3A23 protein levels were essentially at or below the limit of detection for microsomal samples from animals treated with vehicle but were strongly induced by treatment of the rats with pregnenolone 16α-carbonitrile, clotrimazole, troleandomycin or nifedipine. CYP2B was only strongly induced by clotrimazole and CYP4A was not induced by any of the treatments (Fig. 5b).

In order to determine if nifedipine had effects on cholesterol levels in lipid membranes other than the microsomes, pooled membrane fractions from the rest of the liver were examined. The first of these fractions, the NP40-soluble, represents the fraction of the 10,000 g pellet that is soluble in low levels of nonionic detergent. In contrast, the NP40-insoluble fraction contains lipids that are refractory to resuspension in low levels of nonionic detergent. Treatment with nifedipine decreased cholesterol levels in the NP40-soluble fraction but increased cholesterol levels in the insoluble fractions (Fig. 6a).

In order to determine if nifedipine treatment altered additional forms of cholesterol, we also examined levels of total cholesterol in the various subcellular fractions. Similar to unesterified cholesterol, nifedipine treatment decreased total cholesterol levels in microsomes, but increased levels in the NP40-insoluble fraction (Fig. 6b). In contrast to unesterified cholesterol, levels of esterified cholesterol were increased in the NP40-soluble fractions. Although we did not measure levels of esterified cholesterol in the absence of unesterified cholesterol, simple mass balance considerations indicated that esterified cholesterol accounted for the majority of the total cholesterol fraction. As such, these results indicate that levels of esterified cholesterol were decreased in the microsomes but were increased in the crude membrane fractions from the rest of the liver.
**DISCUSSION**

PXR is a central regulator of CYP3A and other enzymes involved in the metabolism of cholesterol precursors and products in the liver\(^6\). Since many drugs are activators of PXR, we speculated that these drugs could have unexpected effects on hepatic cholesterol metabolism. In this study, we found that treatment of rats with nicardipine, a PXR activator, lowered unesterified and esterified cholesterol levels in liver microsomes relative to the total protein levels in this fraction. Since inducers of CYP3A commonly increase the total mass of microsomal protein recovered per gram of liver, one possibility is that normalizing the cholesterol levels to the protein content results in an apparent false dilution of the cholesterol concentration in this fraction. However, several lines of evidence argue against this conclusion. It was previously demonstrated that treatment with nicardipine results in a proportionate increase in phospholipid and protein in liver microsomes\(^{23}\). Since cholesterol only accounts for \(-6\%\) of the lipid content in the endoplasmic reticulum\(^{27}\), the overall effect of treatment with PXR activators is a proportionate increase of both the lipid and protein components of the endoplasmic reticulum rather than a preferential increase in protein. Secondly, the increased recovery of microsomal protein could only account for \(50\%\) or less of the decrease in microsomal cholesterol content observed in response to troleandomycin, nifedipine or nicardipine treatments, even if one incorrectly assumes that there is
not a proportionate increase in the lipid component. Finally, the changes in cholesterol levels in membrane fractions other than microsomes occurred without opposite changes in protein recovery from these fractions. Therefore, it is clear that the effects on hepatic cholesterol observed in these studies were the result of broad changes in hepatic cholesterol processing.

Nicardipine treatment generally increased levels of esterified and unesterified cholesterol in crude membrane fractions. Since the large majority of hepatic cholesterol was located outside the microsomal fraction, it was concluded that nicardipine treatment increased total hepatic cholesterol levels. Cholesterol in the insoluble fraction has been associated with lipid rafts and specific cell-signaling proteins. In addition, the cholesterol content of lipid membranes is known to be an important factor in protein function and activity. Therefore, the large changes in the membrane lipid composition observed in this study suggests that treatment with agents that induce P450 could have broad effects on protein and cellular function.

A series of structurally distinct CYP3A23 inducers decreased microsomal cholesterol levels. DMSO was the only P450 inducer tested that failed to alter microsomal cholesterol levels. DMSO, however, stabilizes P450 levels without a corresponding effect on the mRNA levels and therefore is not likely to activate the PXR. Similarly, although trolessomycin was effective in suppressing microsomal cholesterol levels, it is unlikely that this effect was the result of increased CYP3A activity. This conclusion is based on evidence that even though trolessomycin transcriptionally induces CYP3A23 expression, it is an efficacious mechanism-based inhibitor of CYP3A activity.

Overall, this study provides the first clear evidence that treatment with a variety of drugs known to induce P450 expression also alters cholesterol processing within the liver. The most likely explanation for this effect is that these drugs activate one or more transcription factors that regulate proteins involved in cholesterol metabolism. The data presented here suggest that PXR may be a factor in this process. Even so, it is possible that other receptors that regulate hepatic cholesterol, such as the farnesoid X receptor or the liver X receptor or even cell-signaling events stimulated by P450 generation of reactive oxygen species may be important. Further studies are also needed to determine if the subcellular changes in cholesterol distribution are due to changes in synthesis, intracellular trafficking or extracellular release of cholesterol, cholesterol esters or bile acids. Regardless of the molecular mechanism involved in this process, these effects on cholesterol could have broad implications for a variety of drugs that induce P450 by receptor activation.

An understanding of the processes involved could potentially lead to alternative targets for treating hypercholesterolemia or other diseases.

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REFERENCES


